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Inhibition of the glucosyltransferase activity of clostridial Rho/Ras-glucosylating toxins by castanospermine

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Abstract **Castanospermine was identified as an inhibitor of the Rho/Ras-glucosylating *Clostridium sordellii* lethal toxin and *Clostridium difficile* toxin B. Microinjection of castanospermine into embryonic bovine lung cells prevented the cytotoxic effects of toxins. The crystal structure of the glucosyltransferase domain of *C. sordellii* lethal toxin in complex with castanospermine, UDP and a calcium ion was solved at a resolution of 2.3 Å. The inhibitor binds in a conformation that brings its four hydroxyl groups and its N-atom almost exactly in the positions of the four hydroxyls and of the ring oxygen of the glucosyl moiety of UDP–glucose, respectively.**

Keywords: Glucosyltransferase; Enzyme inhibitors; Crystal structure; *Clostridium difficile* toxin B; *Clostridium sordellii* lethal toxin

1. Introduction

Clostridial glucosylating toxins comprise a family of 250–308 kDa exotoxins, which inactivate low molecular mass GTPases of the Rho and Ras subfamilies of eukaryotic target cells by mono-*O*-glucosylation [1,2]. Several important virulence factors belong to this toxin family, including *Clostridium difficile* toxins A and B, which cause antibiotics-associated diarrhea and pseudomembranous colitis [3]. Other members of this toxin family are the lethal toxin and hemorrhagic toxin from *C. sordellii* and *C. novyi* α-toxin. Whereas *C. novyi* α-toxin uses UDP-*N*-acetylglucosamine as a co-substrate, all other glucosylating toxins utilize UDP–glucose as sugar donor [4]. The toxins differ slightly in their protein substrate specificity. Whereas *C. difficile* toxins A and B, *C. sordellii* hemorrhagic toxin and *C. novyi* α-toxin glucosylate Rho proteins, including RhoA, B and C, Rac and Cdc42, the lethal toxin of *C. sordellii* modifies Ras subfamily proteins, including Ras, Ral and Rap also Rac but not RhoA [1,5,6]. Glucosylation of Rho/Ras proteins occurs at Thr35 (e.g., in Ras/Rac) or Thr37 (e.g., in RhoA), a modification, which inactivates the GTPases and inhibits downstream signaling pathways [1].

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The toxins consist of at least four domains having a receptor binding domain at the C-terminus and the glucosyltransferase domain at the N-terminus. The middle part appears to be responsible for processing and translocation of the toxins. Recently, the crystal structures of the glucosyltransferase domains of toxin B, lethal toxin and α-toxin, which are translocated into the cytosol of target cells, were solved [7,8]. These studies showed that the toxins belong to the GT-A family of glycosyltransferases.

Here we report that the glucosidase inhibitor castanospermine inhibits the catalytic domain of the toxins. With the presentation of the 3D-structure of castanospermine in a complex with lethal toxin of *C. sordellii* we provide major insights into the inhibiting mechanism of the compound.

2. Materials and methods

2.1. Materials

UDP–[^{14}C]glucose (287.4 mCi/mmol) was obtained from Perkin–Elmer Life Science, castanospermine from Tocris Biosciences, deoxynojirimycine (DNJ) and *N*-butyl-deoxynojirimycine (NB-DNJ) from Sigma. Toxin B from *C. difficile* VPI 10463 and lethal toxin (LT) from *C. sordellii* 6018 were purified as described elsewhere [9].

2.2. Expression of recombinant proteins

Toxin fragments (LT546 amino acids 1–546 of *C. sordellii* lethal toxin) and Rac1 were expressed and purified as glutathione S-transferase fusion proteins as previously reported [10,11].

2.3. Glucosylation reaction

Rac (8.7 μM) was incubated with the indicated concentrations of recombinant toxin fragments and 10 μM UDP–[^{14}C]sugars in a buffer, containing 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$ and 1 mM $MnCl_2$ for 15 min at 30 °C (20 μl total volume). Labeled proteins were analyzed by SDS–PAGE followed by phosphorimaging (Molecular Dynamics/GE Healthcare, Freiburg, Germany). Quantification was performed with ImageQuant (Molecular Dynamics).

2.4. UDP–sugar hydrolase reaction

UDP–sugar hydrolysis was performed as described [11]. Toxin fragments were incubated with 20 μM UDP–[^{14}C]sugar and 80 μM unlabeled UDP–sugar in a buffer, containing 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$ and 100 μM $MnCl_2$ for 30 min at 30 °C. Samples (1 μl) were subjected to PEI (polyethyleneimine)-cellulose thin layer chromatography (Merck, Darmstadt, Germany) (mobile phase: 0.2 mM LiCl) to separate the hydrolyzed sugar from UDP–sugar. The plates were dried and analyzed by PhosphorImager analysis.

2.5. Microinjection studies

For microinjection, embryonic bovine lung (EBL) cells were seeded on CELLocate coverslips (Eppendorf, Hamburg, Germany) at about 10^4 cells/dish and cultivated for 24 h in Dulbecco's modified Eagle's

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medium supplemented with 10% fetal calf serum in humidified 5% CO_2 at 37 °C. Microinjection of castanospermine or buffer (Tris 50 mM) was performed with the microinjector 5242 and micromanipulator 5171 from Eppendorf.

2.6. Structure analysis and refinement

Crystals were grown using the hanging drop method at 293 K. The crystallization condition was 100 mM Tris–HCl pH 8.5, 20% (w/v) PEG 4000, 200 mM $CaCl_2$. The drop contained 3.7 µl 8.8 mg/ml

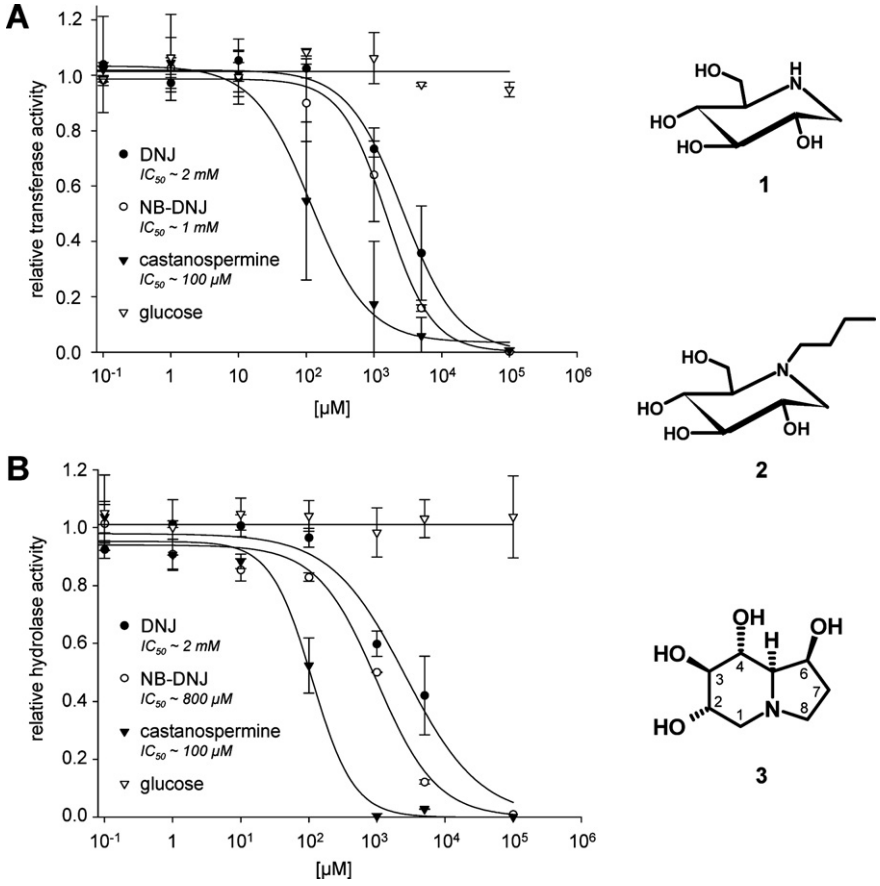

Fig. 1. Inhibition of the enzyme activity of *C. sordellii* lethal toxin by transition state mimicking compounds. (A) Inhibition of glucosylation of Rac. The catalytic domain of lethal toxin (1 nM) was incubated with recombinant Rac and UDP–[^{14}C]glucose at increasing concentrations of glucose, deoxynojirimycine (DNJ) (1), *N*-butyl-deoxynojirimycine (NB-DNJ) (2) and castanospermine (3), respectively. Radiolabeled proteins were analyzed by SDS–PAGE and phosphorimaging. (B) Inhibition of the UDP–sugar hydrolase reaction. The catalytic domain of lethal toxin (300 nM) was incubated with radiolabeled UDP–[^{14}C]glucose. Cleavage of UDP–glucose was analyzed by PEI-cellulose thin layer chromatography and phosphorimaging. IC_{50} values are indicated. Data are shown in means ± S.D. (n = 3).

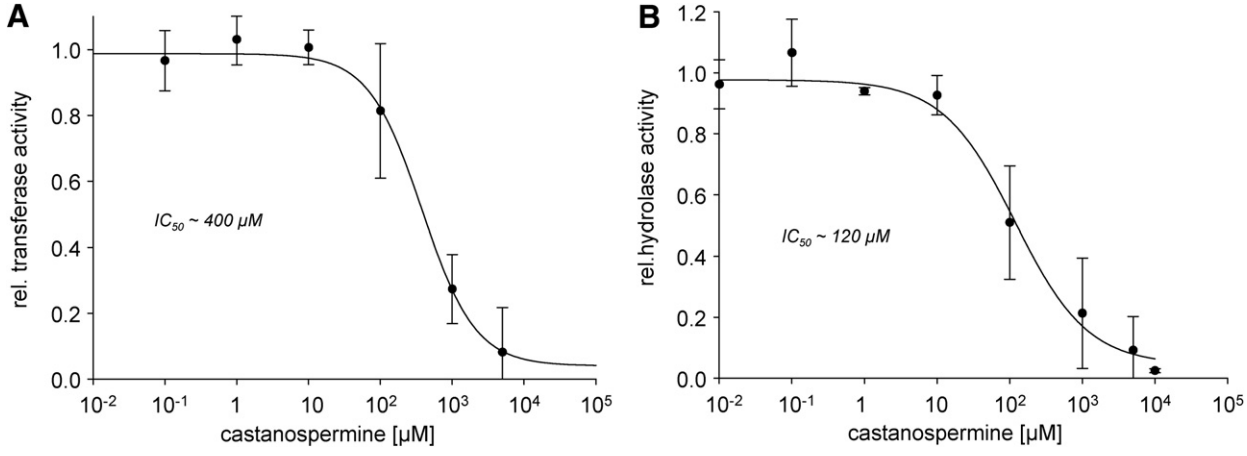

Fig. 2. Inhibition of the enzyme activity of *C. difficile* toxin B by castanospermine. (A) In vitro glucosylation of Rac by *C. difficile* toxin B (5 nM) at increasing concentrations of castanospermine with UDP–[^{14}C]glucose. Radiolabeled proteins were analyzed by SDS–PAGE and autoradiography. (B) UDP–glucose hydrolase activity of *C. difficile* toxin B (300 nM) was determined with radiolabeled UDP–glucose. Cleavage products were analyzed by PEI-cellulose thin layer chromatography and phosphorimaging. IC_{50} values are indicated. Data are shown in means ± S.D. (n = 6).

protein solution, 1 μl 100 mM spermidine and 5.1 μl reservoir buffer. The crystals reached final sizes up to 2000 μm × 250 μm × 50 μm. One crystal was soaked for 10 min with 2 mM UDP and 5 mM castanospermine. After 1 min soaking in reservoir buffer containing 30% (v/v) ethyleneglycol, the crystal was flash-frozen in liquid nitrogen for X-ray data collection. The data were collected at the SLS (beamline X06SA, Villigen, Switzerland). Data processing were done with XDS [12] (Supplementary Table). Castanospermine was modeled into the electron density. The model was refined with REFMAC5 [13]. Water molecules were added automatically with ARP/wARP [14]. Manual building was done using COOT [15]. The ligand was created with PRODRG [16]. Structure validation was done with PROCHECK [13]. Figures were prepared using POVScript+ [17] and POVRAY (http://www.povray.org).

3. Results

We tested several azasugar derivatives, which are known to be potent glycosidase inhibitors, for their potency to inhibit the glucosyltransferase activity of *C. sordellii* lethal toxin. As shown in Fig. 1A, deoxynojirimycine (DNJ) inhibited glucosylation of Rac with an IC_{50} value of about 2 mM. A similar IC_{50} value of 1 mM was obtained for the *N*-alkylated form of DNJ (*N*-butyl-deoxynojirimycine, NB-DNJ). Almost at the same concentrations the compounds inhibited the glycohydrolase activity of the toxins (Fig. 1B) indicating that the azasugars act by inhibiting the catalytic activity and not the interaction between the enzyme and its protein substrate. The plant alkaloid castanospermine is another polyhydroxypyridine analog, which is related to azasugars. This compound has been shown in various studies to inhibit glycosidases [18,19]. As compared to deoxynojirimycine and *N*-butyl-deoxynojirimycine, castanospermine was more potent to inhibit glucosylation of Rac or glycohydrolysis of UDP–glucose by the catalytic domain of lethal toxin ($IC_{50} \sim 100$ μM). Also full length lethal toxin was inhibited by castanospermine with similar potency (IC_{50} for inhibition of glucosylation 180 μM).

In addition, we tested the inhibiting potential of castanospermine on the glucosyltransferase and -hydrolase activity of *C. difficile* toxin B. The compound also blocked the enzyme activity of this toxin (Fig. 2A and B). Several other sugar related analogs (conduritol-B-epoxide (CBE), DMDP ((2R,5R)-bis-(hydroxymethyl)-(3R,4R)-dihydroxypyrrolidine), bromoconduritol (6-bromo-3,4,5-trihydroxycyclohex-1-ene)) were tested without significant inhibition of the toxins activity (data not shown).

To test the inhibiting effect of castanospermine on clostridial glucosylating toxins in intact cells, we studied the influence of castanospermine on toxin-induced cell morphological changes. Lethal toxin and toxin B caused the typical cytotoxic effects on embryonic bovine lung cells, which are characterized by shrinking and rounding-up of the cell body and formation of neurite-like processes. These morphological changes were completely blocked by previous microinjection of castanospermine into the target cells (Fig. 3A and B).

To get further insights into the mechanism of inhibition, we crystallized the enzyme domain of lethal toxin and produced a

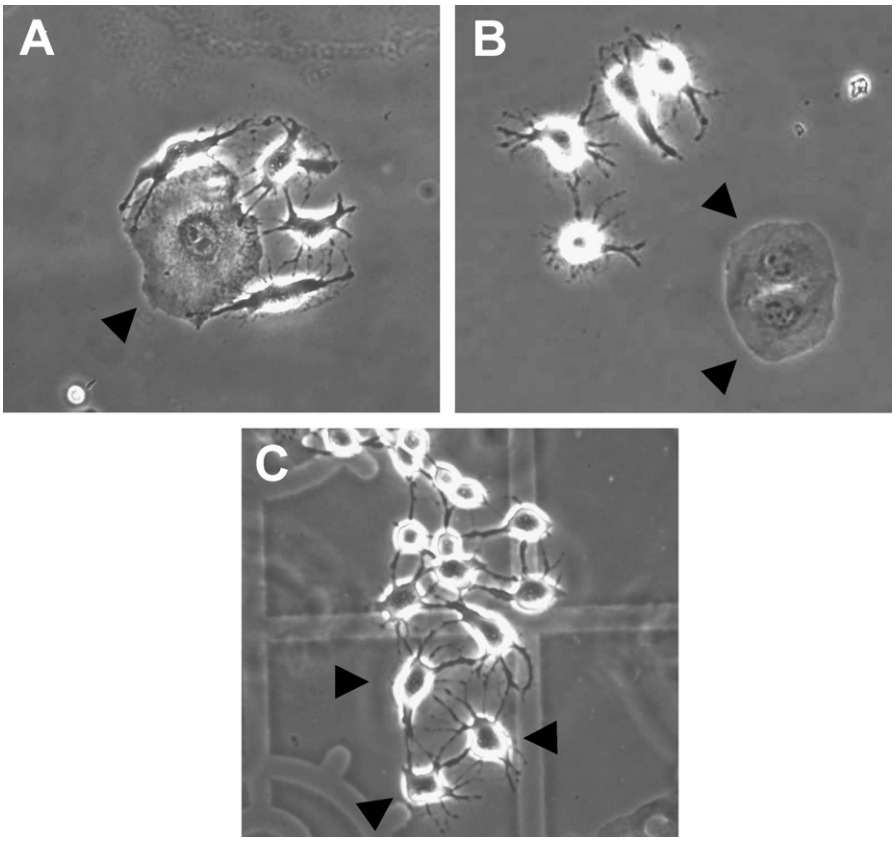

Fig. 3. Protection of cell intoxication by microinjection of castanospermine into EBL-cells. Castanospermine (1 mM, A and B) or control buffer (C) was injected into embryonic bovine lung (EBL) cells (marked with an arrow). Cells were regenerated for 1 h at 37 °C. Afterwards 250 ng/ml *C. sordellii* lethal toxin (A, C) or 100 ng/ml *C. difficile* toxin B (B) were applied to the medium. Photographs were taken 3 h after toxin application.

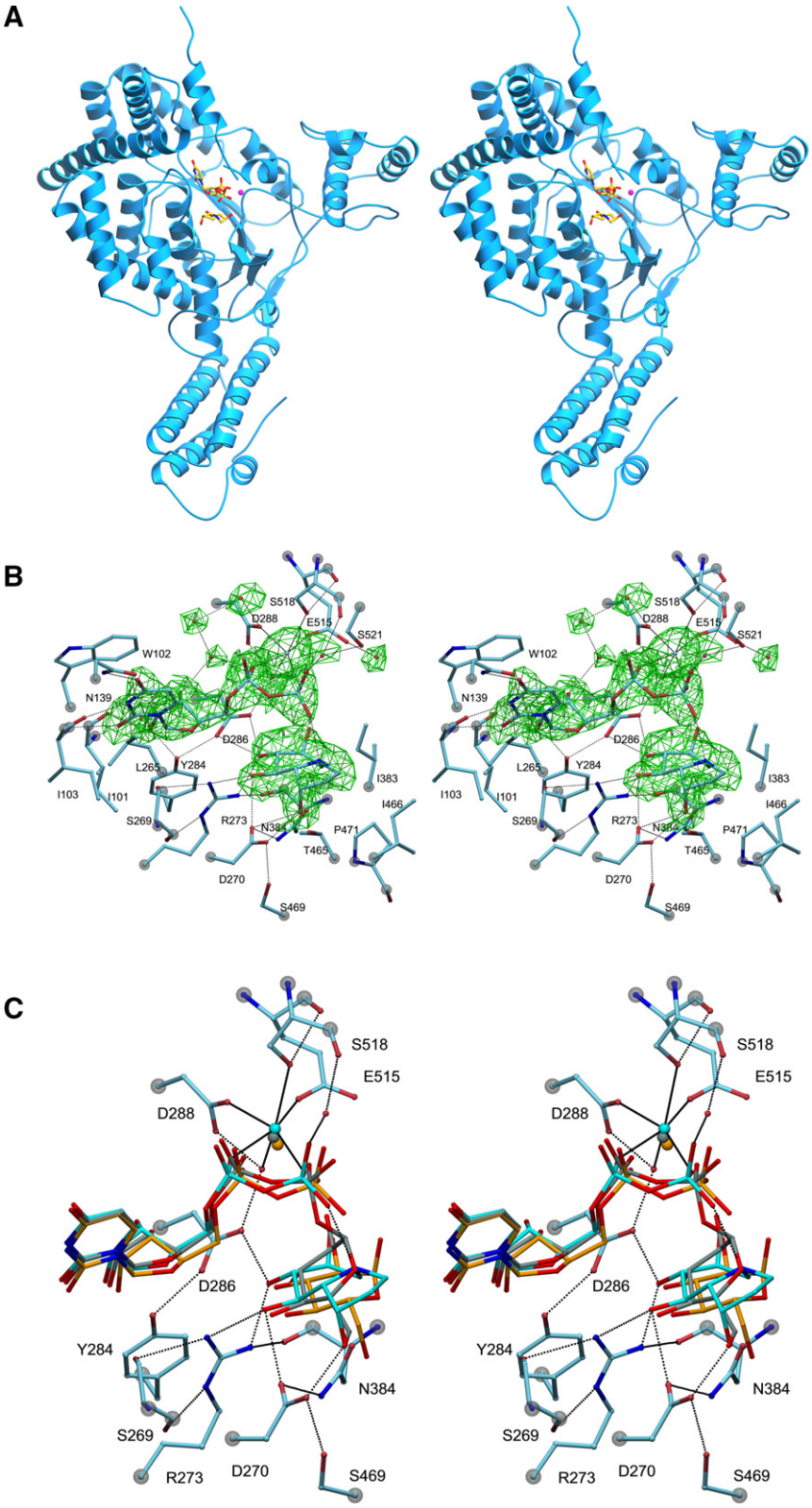

Fig. 4. Binding structure of castanospermine to the catalytic fragment of lethal toxin from *C. sordellii*. (A) Stereoview of the ribbon plot of lethal toxin (blue) with bound UDP and castanospermine (yellow ball-and-stick models) and Ca^{2+} (pink). (B) Stereoview of the binding structures of Ca^{2+}, UDP and the inhibitor in the ($F_o - F_c$)-electron density of a simulated annealing omit map at a 3.0σ contour level. Chain cuts are marked by halos. Hydrogen bonds are dotted lines. Some water molecules are shown in red. (C) Stereoview of the binding structure of Ca^{2+}, UDP and castanospermine in lethal toxin (cyan) superimposed with the binding structures of Mn^{2+} and UDP–glucose in lethal toxin (grey) [8] as well as Mn^{2+}, UDP and glucose in toxin B of *C. difficile* (orange) [7]. The N-atom of the inhibitor is at distances of 0.4 Å and 1.2 Å to the O5″ and C1″ atoms of UDP–glucose, respectively.

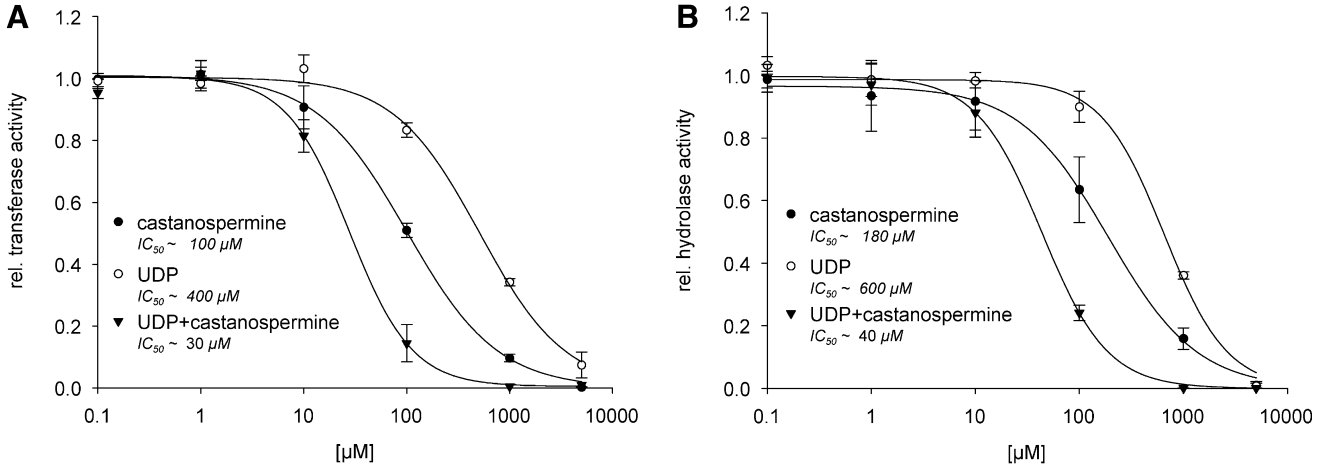

Fig. 5. Enhancement of the inhibiting effect of castanospermine in the presence of UDP. (A) Inhibition of glucosyltransferase activity of the catalytic domain of lethal toxin (1 nM) was studied at increasing concentrations of castanospermine (•), UDP (○) and an equimolar mixture of castanospermine and UDP (▼) with Rac and UDP–[^{14}C]glucose. Radiolabeled proteins were analyzed by SDS–PAGE and phosphorimaging. (B) Inhibition of UDP–glucose hydrolysis. UDP–glucose hydrolysis by the catalytic domain of lethal toxin (300 nM) was determined at increasing concentrations of castanospermine (•), UDP (○) and an equimolar mixture of castanospermine and UDP (▼). Cleaved radiolabeled products from UDP–[^{14}C]glucose were separated by PEI-cellulose thin layer chromatography, analyzed by autoradiography and quantified with ImageQuant. Data are shown in means ± S.D. (n = 3).

crystalline complex with castanospermine. Whereas attempts to crystallize the complex in the presence of manganese salts failed, we obtained suitable crystals in the presence of calcium and UDP (Supplementary Table). The structure of the complex between lethal toxin and Ca^{2+}, UDP as well as castanospermine was solved by the molecular replacement method at 2.3 Å resolution (Fig. 4A). The polypeptide conformation was closely similar to that of a complex between lethal toxin and Ca^{2+} together with UDP–glucose [8]. The *rmsd* of the Cα atoms was 0.3 Å. The Ca^{2+} coordination differs with respect to the identified water molecules [8]. The castanospermine conformation is well-established by the difference electron density (Fig. 4B). In Fig. 4C, the binding mode of the inhibitor is superimposed on UDP–glucose as bound in a catalytically competent Mn^{2+} complex of lethal toxin [8] and in a complex between the toxin B of *C. difficile* [7] and Mn^{2+}, UDP as well as glucose. This superposition demonstrates that the four hydroxyls of the inhibitor are almost exactly in the positions of the respective hydroxyls of UDP–glucose and of glucose. The non-polar ethylene bridge between the 6-hydroxyl group and the N-atom of castanospermine is in a non-polar pocket formed by Ile383, Thr465, Ile466 and Pro471, which is an appropriate place. The N-atom is very close to the pyranose ring oxygen of UDP–glucose and forms a salt-bridge to the β-phosphoryl group of UDP suggesting that the presence of UDP increases the binding strength.

Therefore, we tested whether UDP had any effect on inhibition of lethal toxin-catalyzed glucosylation of Rac by castanospermine. UDP alone had only a weak inhibiting activity. By application of both UDP and castanospermine, the inhibition curve was shifted to the left and the IC_{50} value (~30 μM for inhibition of glucosylation) was reduced by about 3-fold (Fig. 5A). A similar result was observed for inhibition of the glycohydrolase activity by castanospermine (Fig. 5B). Again in the presence of UDP, castanospermine was about 3-fold more potent to inhibit glycohydrolase activity than in its absence.

4. Discussion

Inhibitors of clostridial glucosylating toxins are of potential therapeutic value. UDP–mannose has been described as an inhibitor of the action of *C. sordellii* lethal toxin [20]. Its inhibiting potency is in the mM range and its mechanism is not well defined. Here we studied the effects of castanospermine, which is a polyhydroxylated indolizidine alkaloid from the seeds of the black bean tree *Castanospermum australe*, on the glucosyltransferase activities of *C. sordellii* lethal toxin and *C. difficile* toxin B and obtained IC_{50} values of 100–400 μM for the inhibition of glucosylation of Rac. Castanospermine was more potent than the related compounds deoxynojirimycine and *N*-butyldeoxynojirimycine. At the same concentrations, castanospermine inhibited the glucosylhydrolase activity of the toxins, indicating that the enzyme–protein substrate interaction was not blocked. Castanospermine and the related compounds are well known inhibitors of glycosidases [18,19,21]. They inhibit glycosidases of different types with largely different potencies.

It has been suggested that castanospermine and related compounds act as transition state mimicking structures to inhibit glycosidase reaction [18]. Structural basis is most likely its positive charge and the geometric restriction of the bicycle. The mechanism involved in hydrolysis of the glycosidic bond is well understood for most retaining and inverting glycosidases. In many cases, it is suggested that the transition state involves an oxocarbenium cation-like transition state [22] with distortion of the substrate sugar into a boat or half-chair conformation to lower the energy barrier of the transfer reaction. Accordingly, it has been reported for the β-glucosidase *Tm*GH1 from *Thermotoga maritime* that castanospermine is bound in a boat conformation [18], actually different from the structure seen in the small molecule crystal structure of castanospermine [23]. The binding structure of castanospermine in lethal toxin (Fig. 4) corresponds closely to that of UDP–glucose in a catalytically competent complex [8]. In order to fit the four hydroxyl positions of UDP–glucose, the

inhibitor assumes an unusual conformation. Its positively charged N-atom is located almost exactly at the position where the putative transient positive charge of the carboxonium ion is expected. The observed binding structure suggests that the 6-epimer of castanospermine binds more effectively than the inhibitor itself. Moreover, it suggests that additions at atoms C1 and C8 may differentiate between different protein substrates. Even more importantly, such additions may avoid side-reactions with other glycosyltransferases.

The observation that UDP increased the potency of castanospermine is well-explained by the structure, because the β-phosphate of UDP forms an energetically favorable salt-bridge with the nitrogen of castanospermine (Fig. 4C). Since the nitrogen atom of castanospermine is located within 1 Å at the C1″ and O5″ positions of the substrate UDP–glucose (Fig. 4C), the observed castanospermine binding structure suggests that such a salt-bridge may be an intermediate of the glycosyltransfer reaction, as recently proposed in the circular electron transfer mechanism [8]. Consequently, Fig. 4C supports the circular mechanism that suggests the formation of such a salt-bridge as the initial step, which is then followed by the nucleophilic attack of the sugar-accepting atom [8]. Taken together, our studies show that the glucosidase inhibitor and transition state mimic castanospermine potently inhibits Rho/Ras-GTPases glucosylating toxins. Moreover, castanospermine is a potential lead structure for the development of therapeutic inhibitors of clostridial glucosylating toxins.

Protein accession code: The data were deposited in the Protein Data Bank under accession code 2VL8.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.05.025.

References

[1] Just, I., Selzer, J., Wilm, M., Von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995) Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature 375, 500–503.
[2] Voth, D.E. and Ballard, J.D. (2005) Clostridium difficile toxins: mechanism of action and role in disease. Clin. Microbiol. Rev. 18, 247–263.
[3] Kelly, C.P. and LaMont, J.T. (1998) Clostridium difficile infection. Annu. Rev. Med. 49, 375–390.
[4] Selzer, J., Hofmann, F., Rex, G., Wilm, M., Mann, M., Just, I. and Aktories, K. (1996) *Clostridium novyi* α-toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins. J. Biol. Chem. 271, 25173–25177.
[5] Just, I., Selzer, J., Hofmann, F., Green, G.A. and Aktories, K. (1996) Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. J. Biol. Chem. 271, 10149–10153.
[6] Popoff, M.R., Chaves, O.E., Lemichez, E., Von Eichel-Streiber, C., Thelestam, M., Chardin, P., Cussac, D., Chavrier, P., Flatau, G., Giry, M., Gunzburg, J. and Boquet, P. (1996) Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. J. Biol. Chem. 271, 10217–10224.
[7] Reinert, D.J., Jank, T., Aktories, K. and Schulz, G.E. (2005) Structural basis for the function of *Clostridium difficile toxin* B. J. Mol. Biol. 351, 973–981.
[8] Ziegler, M.O.P., Jank, T., Aktories, K. and Schulz, G.E. (2008) Conformational changes and reaction of clostridial glycosylating toxins. J. Mol. Biol. 377, 1346–1356.
[9] Just, I., Selzer, J., Hofmann, F. and Aktories, K. (1997) *Clostridium difficile Toxin* B as a Probe for Rho GTPases in Bacterial Toxins – Tools in Cell Biology and Pharmacology, Chapman & Hall, Weinheim, pp. 159–168.
[10] Jank, T., Reinert, D.J., Giesemann, T., Schulz, G.E. and Aktories, K. (2005) Change of the donor substrate specificity of *Clostridium difficile* toxin B by site-directed mutagenesis. J. Biol. Chem. 280, 37833–37838.
[11] Just, I., Wilm, M., Selzer, J., Rex, G., Von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995) The enterotoxin from *Clostridium* difficile (ToxA) monoglucosylates the Rho proteins. J. Biol. Chem. 270, 13932–13936.
[12] Kabsch, W. (1993) Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell contents. J. Appl. Cryst. 26, 795–800.
[13] Collaborative Computational Project N.4 (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D: Biol. Crystallogr. 50, 760–763.
[14] Perrakis, A., Morris, R. and Lamzin, V.S. (1999) Automated protein model building combined with iterative structure refinement. Nat. Struct. Biol. 6, 458–463.
[15] Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D: Biol. Crystallogr. 60, 2126–2132.
[16] Schuttelkopf, A.W. and van Aalten, D.M. (2004) PRODRG: a tool for high-throughput crystallography of protein–ligand complexes. Acta Crystallogr. D: Biol. Crystallogr. 60, 1355–1363.
[17] Fenn, T.D., Ringe, D. and Petsko, G.A. (2003) POVScript+: a program for model and data visualization using persistence of vision ray-tracing. J. Appl. Cryst. 36, 944–947.
[18] Gloster, T.M., Meloncelli, P., Stick, R.V., Zechel, D., Vasella, A. and Davies, G.J. (2007) Glycosidase inhibition: an assessment of the binding of 18 putative transition-state mimics. J. Am. Chem. Soc. 129, 2345–2354.
[19] Cutfield, S.M., Davies, G.J., Murshudov, G., Anderson, B.F., Moody, P.C., Sullivan, P.A. and Cutfield, J.F. (1999) The structure of the exo-beta-(1,3)-glucanase from *Candida albicans* in native and bound forms: relationship between a pocket and groove in family 5 glycosyl hydrolases. J. Mol. Biol. 294, 771–783.
[20] Humeau, Y., Popoff, M.R., Kojima, H., Doussau, F. and Poulain, B. (2002) Rac GTPase plays an essential role in exocytosis by controlling the fusion competence of release sites. J. Neurosci. 22, 7968–7981.
[21] Rhinehart, B.L., Robinson, K.M., King, C.H. and Liu, P.S. (1990) Castanospermine-glucosides as selective disaccharidase inhibitors. Biochem. Pharmacol. 39, 1537–1543.
[22] Vasella, A., Davies, G.J. and Bohm, M. (2002) Glycosidase mechanisms. Curr. Opin. Chem. Biol. 6, 619–629.
[23] Hempel, A., Camerman, N., Mastropaolo, D. and Camerman, A. (1993) Glucosidase inhibitors: structures of deoxynojirimycin and castanospermine. J. Med. Chem. 36, 4082–4086.